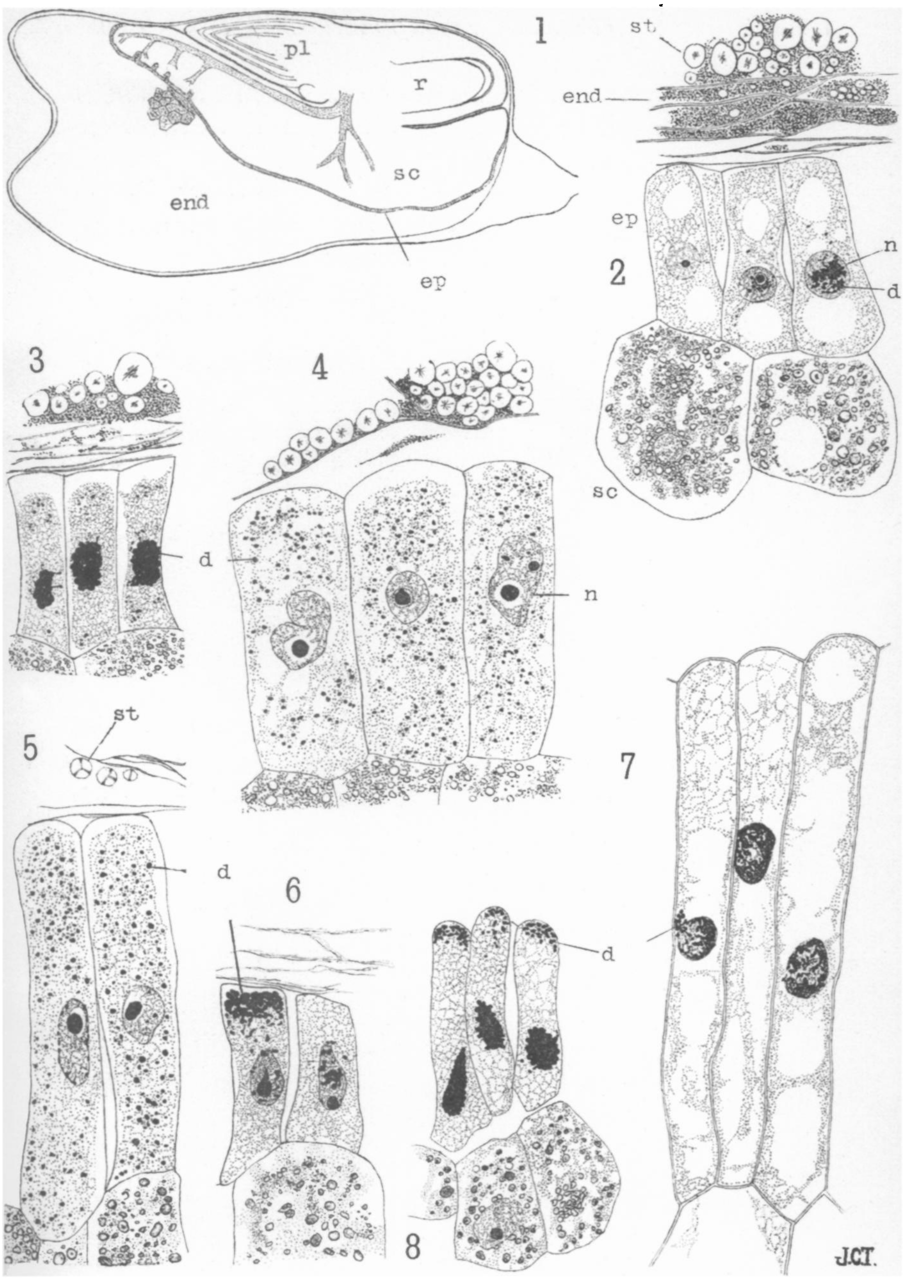

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Cytological Changes accompanying the Secretion of Diastase

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(WITH PLATE 20)

From the chemical standpoint, a large amount of work has already been done on the secretion of diastase in the Gramineae. For these researches we are principally indebted to Morris and Brown. These authors have determined with great care the cells concerned in its formation, the amount secreted and its method of attack on the endosperm. Many botanists, too, have described and figured the pitting and ultimate dissolution of the starch grains in the endosperm during the progress of germination. No serious attempt, however, has been made to determine the nature and sequence of the changes occurring in the diastase-secreting cells during the elaboration of the enzyme. This paper, accordingly, embodies an attempt to throw some light on the following questions :

1. In what part of the diastase-producing cells is the ferment produced ? Is it in the nucleus or in the cytoplasm ?

2. How soon after the beginning of germination is diastase secreted ? Is it formed intermittently or is it a continuous secretion ?

Little attention has been paid to the action of the enzyme on the endosperm, except in so far as it gave evidence that diastase had been discharged from the cells.

As maize has been found to be in many ways more favorable for study than barley, it has largely furnished the material for the present paper. The latter, however, has been of value from a comparative standpoint. I take pleasure in acknowledging my

indebtedness to Professor Francis E. Lloyd for the suggestion of the research and kindly criticism during its progress.

Methods.—The seeds were soaked over night in water to soften the coats, and were then placed between layers of filter paper in a moist chamber. The embryos at different stages of growth were cut from the grain together with that part of the endosperm in immediate contact with the scutellum. The first series was made at time intervals. The embryos were fixed approximately every 12 hours for two days, and then at 24-hour intervals. This was found to be unsatisfactory as the rate of growth in different seeds varied greatly, and thus the chances of obtaining a sequence of stages was lessened. Accordingly, a series from the beginning of germination to five days was secured with the rate of growth as a guide, and without reference to time intervals. The length of the radicle and plumule served as an indicator. The best fixation was effected by the use of sublimate acetic 2 per cent., or of alcoholic acetic (70 per cent. alcohol 2 parts and glacial acetic acid 1 part). Flemming's fluid caused excessive blackening. Iron haematoxylin proved to be by far the most satisfactory stain, either alone or followed by Congo red as a counter stain. Auerbach's method, also, was of some value. The sections were cut for the most part 5 μ in thickness.

I. DESCRIPTION OF SEED. (PL. 20, F. 1)

Without entering upon a description of the various seed coats, a kernel of corn may be said to consist of two distinct elements—the endosperm and the embryo. The former occupies the larger part of the seed and partly clothes the adjacent embryo. The latter is on the furrowed flattened side of the kernel. It is thickest at the end attached to the cob, and gradually slopes to a point reached at about four fifths the length of the grain. Between the axis of the plantlet and the endosperm there is a thickened shield-like expansion of the hypocotyl, supposed by many to be the homologue of the cotyledon, and known as the scutellum. This is the special organ of absorption. The endosperm consists of large cells filled with starch grains imbedded in a matrix of proteid matter. Several layers of crushed and empty cells separate these well-filled cells from the scutellum. As these cells

have been emptied by the pressure of the growing scutellum during the development of the seed, we find them most numerous where the scutellum is thickest. Directly adjoining the flattened empty cells is the epidermal layer of the scutellum, the cells of which manufacture the " diastase of secretion."

These secretory cells are found at every point where the scutellum is in contact with the endosperm. At the region of the tip this secretory epithelial layer dips down at frequent intervals into the scutellum. The convolutions so produced secure a larger surface of secretion where there is greatest need for the enzyme; for the endosperm is thickest at this point and in front of the embryo. Immediately below the epithelial cells are the large loosely arranged isodiametric cells of the scutellum. The larger cells are near the central part of the scutellum. Here, too, the cell walls are thicker than at the periphery. The young shoot itself, is closely connected with the lower central portion of the scutellum. From the hypocotyledonary part there proceeds into the scutellum a branch of the vascular system which soon divides into two parts. One of these runs directly upward, and the other toward the tip of the scutellum. They both give rise to many small branchlets, but none of these find their way into the heel of the scutellum.

The diastase-secreting cells are columnar, and at the beginning of germination are about three times as long as broad (*f. 2*). The bases of the cells rest on the scutellar parenchyma, and their ends are in close apposition to the endosperm. The cell walls are extremely thin and delicate, thus permitting a free osmotic interchange. The protoplasm in early stages is finely reticulated, but becomes somewhat coarser as germination progresses. The nucleus varies greatly in its position. Although it is found as a rule near the center, it may not infrequently occur at the base or near the outer end of the cell. It is slightly elliptical with its long dimension parallel to that of the cell. The nuclear wall is very delicate. The chromatin, in the form of small granules, lies scattered in the linin network. The large, deeply staining nucleolus always lies in a vacuole. Two such nucleoli very commonly occur. Although as a rule round, a knot like projection is sometimes present. The cells in the central part of the scutellum are especially

heavily loaded with nutritive matter, which consists, for the most part, of aleurone grains and oil globules. In corn, even at the beginning of germination, starch grains are commonly found in these cells. In barley, such grains, according to Morris and Brown, are not present in the scutellum until the endosperm shows signs of depletion. Nearly all the starch grains, wherever found, are cracked because of the dehydration of the resting period.

II. ORIGIN OF DIASTASE

1. *Previous Accounts.*—It will be sufficient, for our present purpose, to point out the following pertinent facts in regard to diastase, which have been established principally through the researches of Morris and Brown.*

(*a*) There are two varieties of diatase.—One is an amylohydrolytic enzyme occurring in buds, leaves and other regions, which acts on the transitory starch grains found in the cells of these tissues. It does not act on starch paste nor pit starch granules, but dissolves them evenly.

The other is the enzyme secreted by the columnar epithelial cells in the seeds of the Gramineae. This dissolves starch paste and corrodes starch granules.

The former has been called by Morris and Brown the "diastase of translocation" and the latter the "diastase of secretion."

(*b*) This diastase of secretion begins to be formed soon after the beginning of germination and continues until the endosperm is depleted.

(*c*) The first evidence of the formation of the ferment is the appearance of granules in the columnar epithelial cells of the scutellum.

(*d*) Soon after the appearance of these granules the cells walls of the endosperm nearest the scutellum begin to be dissolved and the contained starch grains show evidence of pitting.

(*e*) Concomitant with the above-mentioned phenomena starch grains appear in the cells of the scutellum immediately below the secreting epithelial cells.

* For a detailed description of the work that has already been done on the origin of diastase and its action on the endosperm, the reader is referred to the original papers or to the chapter on Diastase in Reynold Green's Fermentation.

(*f*) If a small quantity of cane sugar is added to the starch, the latter is protected in a remarkable manner. There is no sign of pitting of the starch grains as long as any sugar remains unabsorbed by the embryo. "Thus the secretion of active diastase by the epithelium may be regarded to some extent as a starvation phenomenon."

(*g*) Lastly, in addition to the starch-dissolving enzyme (amylohydrolyst), there is present in the growing plantlet a cellulose-dissolving enzyme (cytohydrolyst). Both these enzymes are secreted by the columnar ("absorptive") epithelium.

2. *Observations.*—In previous accounts of the secretion of diastase, the statement that the epithelial cells at the beginning of germination are filled with granules seems to be the nearest approach to an accurate description of its origin. This is true as far as it goes. I have attempted to trace these diastase granules to their ultimate origin and I think that there is evidence for the conclusion that in corn and barley and probably in all the Gramineae, "diastase of secretion" arises in the nucleus of the epidermal cells of the scutellum.

My earliest preparations of corn are sections of a kernel that had been soaked in water just long enough to soften the outer coats. The protoplasm of the columnar cells is found to be much shrunken owing to the dry condition of the resting seed. The nuclei for the most part stain very deeply, either wholly, or in several places, although here and there are nuclei which are clear. By the use of a high power this dark staining matter is seen to be distinctly granular (*f. 2*). It stains fully as intensely as chromatin. These granules are often collected in little bunches. Sometimes a string of them lie about the vacuole of the nucleus. The granules seem to arise along the course of the linin network, for many of them may be seen scattered throughout the reticulum. Even at this early stage the nucleus is often so packed with granules that it stains a uniform black and the granular constitution of the mass is shown only by its corrugated edge. In cases where the epidermal cell is double, *i. e.*, where there is an inner and an outer cell, the nuclei of both produce these granules.

Passing now to a kernel that has been placed a little longer in conditions favorable to germination, we find the cytoplasm filled

with these granules and the nucleus empty. Cells that are in an intermediate condition make it clear how this change has taken place. A careful examination of a heavily loaded nucleus will often show a row of granules in the shape of a fine knotted thread extending from the black staining mass in the nucleus through the nuclear membrane and out into the cytoplasm of the cell. There may be several of these threads varying in thickness, but always composed of distinct granules (*f. 3*). Sometimes a thread ends in the cytoplasm in a bunch of granules. As a rule the breaks in the nuclear wall, through which the granules find a point of exit, are very minute, but, in later stages especially, they may be quite extensive (*f. 6*).

In the majority of cases these ruptures seem to be brought about by the constantly increasing size of the mass of granules. Since these granules are present in many nuclei in great abundance before germination has begun, the conclusion is inevitable that they were formed before the resting period. On the other hand, it is no less probable that no diastase is secreted from the cell until the beginning of germination, or, to use Matthew's term, up to that time there is merely "hylogenesis." It is worthy of note, however, that the processes going on at the beginning of germination are a direct continuation of those brought to a stop in the developing embryo by the resting period.

An examination of these same columnar cells in a grain that has been placed under conditions favorable for germination for about 18 hours shows a very striking change in their organization. They are so greatly swollen that they are now at least three times their former size (*f. 4*). The nuclei are for the most part entirely devoid of granules. These have become scattered throughout the cytoplasm, in all probability by virtue of the circulation of the cell sap. Many of the granules have increased considerably in size but the large ones are not restricted to any particular region of the cell. This increase in size of the granules lends color to the theory that a "prozymogen" is formed in the nucleus which becomes an active ferment only after certain elements have been added to it from the cytoplasm.

The nuclei themselves are distorted and in some cases the swelling has been so violent that the delicate nuclear wall is rup-

tured and the nucleolus, vacuole and all, is ejected. The absorption of water by the diastase-secreting cells has, without doubt, caused their swollen condition. This condition, however, is very transitory, for at the end of the first day of germination we find these cells only slightly larger than they were before it occurred. After 24 hours the cells have not only regained their original size, but, in most instances, have also lost their granules. Certain cells are to be found here and there in which the granules are massed together at their ends nearest the endosperm, but otherwise the cytoplasm and nuclei are entirely destitute of them- At the end of the first day, too, the endosperm shows evidence of the action of an enzyme. The solvent action, at this stage, is con. fined to the cell walls just outside the diastase-secreting epithelium and to the proteid matter between the starch granules. The coincidence of the disappearance of the dark staining granules from the cells that secreted them, and the dissolution of a part of the endosperm, is a strong proof of the diastatic character of the former. By this time the radicle has broken through the coleorhiza and projects for about 1.5 to 2 mm. from the seed.

At the beginning of the second day of germination the diastase-secreting cells are in a resting condition. There is no increase in size and the nuclei and cytoplasm are clear. The solvent effect of the diastase secreted during the first day is now more apparent. Not only are the cell walls in the neighborhood of the epithelium broken down, but the starch grains themselves are pitted. The method of attack of the diastatic enzyme on starch grains has been frequently described and nothing was observed which calls for special mention.

At the end of the second day the epithelial cells again begin to show signs of activity, and the second period of secretion is inaugurated. Dark staining granules have appeared once more in some of the nuclei. These granules steadily increase in quantity until, as before, they completely fill the nuclei. The cytoplasm, however, remains clear of them, and there is no increase in size of the cells themselves until some time during the third day. After about 72 hours of germination, groups of the diastase-secreting cells increase once more threefold in size and the cytoplasmic reticulum is filled with granules precisely as during the first day

(*f. 5*). All the cells, at a given time, are by no means at the same stage in the elaboration of the enzyme. This is especially the case after the first day. We may say, nevertheless, that the first and second periods of secretion are separated by an interval of from 36 to 48 hours. By this time the plumule has broken through, and the radicle is an inch and a quarter in length. The scutellar cells show now a notable increase in the number and size of the starch grains that they contain. This is of course due to the fact that, by virtue of the solvent action of diastase, the starch of the endosperm is being transported and stored up in the scutellum, where it serves as food for the growing plant. The usual reduction in size follows the swollen condition, and is accompanied by a collecting of the granules at the tips of the cells, and finally by a discharge of the enzyme into the endosperm.

From now on until the complete depletion of the endosperm and the final degeneration of the scutellum there are no sudden changes in the size of the secreting cells. There seems, on the other hand, to be a gradual, but steady, increase in size with a continual production of diastase by each cell independently of the others. During the fourth day the nuclei of many cells became filled again with granules and on the fifth these may frequently be seen breaking through the nuclear membrane and collecting at the tips of the cells directly, without at first becoming scattered through the cell (*f. 6*). At the end of 11 days there are signs of degeneration in some of the epithelial cells. These consist of an abnormal swelling and vacuolization of the cytoplasm, most noticeable at the ends of the cells towards the scutellum. After the lapse of 22 days the cytoplasm is very scanty and ragged. The cells have also become very greatly elongated. They are now at least three and a half times their length at the beginning of germination. The cell walls are thick and stratified. The nuclei still stain very darkly and are probably producing a ferment for the dissolution of what is left of the cell walls of the endosperm. The starch grains have completely disappeared from the endosperm and the scutellar cells are mere skeletons (*f. 7*). The time given, at which various changes in the diastase secreting cells occur, is merely approximate and varies greatly according as the conditions are more or less favorable for growth.

A sufficient number of stages in germinating barley have been studied to make it clear that we have the same series of phenomena here during the secretion of diastase as in maize. The granules arise in the nucleus, they find their way into the cytoplasm, and immediately after their disappearance from the cell we note the action of an enzyme on the endosperm. The collecting of the diastase granules at the tip of the cell nearest the endosperm is especially noticeable in barley (*f. 8*).

Although the iron haematoxylin stain is one of the most useful ones for cytological purposes, there is a common source of error in its employment, which should be carefully guarded against. It has a tendency to stain in a very deceptive way any precipitates that may be present in the cell. Such precipitates are easily mistaken for natural metabolic products. Accordingly Auerbach's method was used as a precautionary measure. It is interesting to note that those stages of the nuclei which stain an intense black with iron haematoxylin, also stain very deeply with methyl green; whereas the nuclei with granule-filled cytoplasm stain very lightly with methyl green. This is another proof that matter is ejected from the nuclei at the same time that granules appear in the cytoplasm.

3. *Comparative.*—Very few examples of secreting cells in plants have been studied with any degree of care. The results of Huie and Schniewind-Thies, accordingly, are of especial interest in this connection. Miss Lily Huie ('96) found that during secretion in *Drosera* the basophile cytoplasm was depleted. The basophile chromatin increased in the meantime greatly in extent, and, after ejection from the nucleus, gave rise to new basophile cytoplasm. After long-continued secretion the basophile cytoplasm was entirely replaced by a scanty eosinophile cytoplasm. It was not determined whether the nuclear, or nucleolar chromatin, was the primary product of the metabolism. In diastase-secreting cells the cytoplasm is not so directly concerned in the formation of the secretory product as appears to be the case in *Drosera*. The only evidence of the possible activity of the cytoplasm in the former is the increase in size of some of the granules scattered through it. Schniewind-Thies ('97), who has made a very thorough study of the nectar cells in a number of plants, finds that,

as a rule, at the height of secretion these cells are much swollen. The nucleus becomes greatly enlarged and distorted, often sending out pseudopodial processes. As a general rule there is a loss of staining power in the nucleus during secretion. In the end the cytoplasm is much shrunken and the nucleus and nucleoli completely disappear. The nectar begins to appear at the end or just outside the end of the cell. In this case the nucleus seems to play a very active rôle and bears a strong resemblance to the behavior of the nucleus during the secretion of diastase. In both there is a final disappearance of the nucleus after the cell has exhausted itself by its long-continued secretions. In both also there is a swelling of the cell during the formation of the secretion. It seems not unlikely that this swelling is brought about by osmosis set up by some substance secreted within the cell at this time. The diastase, being of a proteid nature and consequently of high molecular weight, would not be, even if in solution, as active osmotically as other less complex substances, as for instance, organic salts or acids, which, De Vries has shown, exercise a strong osmotic attraction when present in the cell. Nevertheless the swelling takes place when the nucleus has become completely filled with these granules. A possible explanation of this phenomenon may be that some organic acid is formed during the great metabolic activity of the cell. The fact that diastase, in order to be especially effective as a ferment, must be dissolved in a liquid with a distinct, but not too strong acid reaction, lends support to this hypothesis.

A comparison of the secretory processes in plants with those in the animal kingdom, where the literature is much more complete, is certainly of interest. Mathews ('99) has studied the secreting cells of the pancreas very carefully. He describes the pancreas cell as divided into two zones, a granular inner and a striated outer. The fibrils of the outer zone arise in the chromatin and end in the cytoplasm of the inner (granular) zone. "The chromatin has, then, formed a highly complex substance probably a nucleo-albumin (the cell thread or fibril) which splits into at least two constituents, one of which forms the granules, and the other a reticulum. The granular substance is perhaps further altered and ultimately forms the zymogen." The interesting fact from a

comparative standpoint is that, whereas in diastase-secreting cells the ferment is found directly by the nucleus, in the pancreatic cell the nucleus does not play an active rôle, but indirectly controls the zymogenesis. Nevertheless, in both cases the *nucleus* is the ultimate source of the zymogen granules. Mathews, in agreement with Nussbaum, arrives at the rather sweeping conclusion that the changes in the nuclei of the secreting cells are passive, the nuclei taking no active part in secretion or in zymogenesis. The active part taken by the nucleus in the production of the diastase granules certainly forms a very evident exception to this generalization and is a point in favor of the views of Heidenhain, Korschelt and others, who consider that the changes in position and size of the nuclei during secretion are signs of functional nuclear activity.

There is considerable evidence that chromatin is the synthetic substance of the nucleus and in the elaboration of the metabolic products acts somewhat in the manner of a ferment. In other words it brings about the union of certain elements without entering into the synthesis itself, or becoming changed in its chemical nature. Mathews thinks that the fact of the cell threads ending in the chromatin points to this conclusion. The secretion or rather the hylogenesis of diastase seems to be a case exactly to the point, as here the process is strictly intranuclear. Pekelharing ('95) and Halliburton ('95), working from another viewpoint, have demonstrated that fibrin ferment is a nucleo-proteid, a form of chromatin. That the amylolytic granules, while in the nucleus, stain in the same manner and with the same intensity as chromatin, indicates that they have something in common with the fibrin-ferment. According to Macallum the nuclei of the pancreatic cells have much to do with the formation of the enzyme. His experiments lead him to the conclusion that the chromatin elaborates a substance which he calls prozymogen. This finds its way into the cytoplasm and, combining with certain elements, forms the granules of the enzyme; certainly a very good description of the sequence of stages in the diastase cell.

The wide-spread occurrence of granules in secreting cells is worthy of note. Green ('99) giving quite a number of examples in which this is the case. In the mucous glands of the frog's

tongue there is a clear protoplasmic zone and a granular zone; a serous glandular cell is filled with granules during the resting period; a like phenomenon is seen in the pepsin-secreting cells. According to Ward,* during the secretion of rhamnase by *Rhamnus infectorius* granules appear.

In animal tissues the secretions are, as a rule, intermittent, but in plant tissues, according to Green, they are prolonged and probably only to a slight degree intermittent. This he explains by the fact that, in the animal, digestion is continually repeated, whereas in plants the utilization of reserve materials is a continuous and very gradual process. During the first three or four days, however, in the case of maize and probably all the Gramineae there is clearly an intermittence in the secretion of the enzyme.

The active part taken by the nucleus in secretion in plants, at least, is in complete harmony with the view that it is the metabolic center of the cell. Verworn, in his General Physiology, cites a number of instances in which the nucleus takes an active part in the nutrition and growth of the cell. Although, as he observes, the presence of a membrane generally necessitates the exchange of liquids between nucleus and protoplasm, yet many observers, among them Fromman and Auerbach, "have observed on the part of the nucleus a direct ingestion and extrusion of granules and flakes." The ejection of granules from the nucleus of the diastase-secreting cell is another case to the point.

A general review of the literature on secretion in plants and animals leads one to the conclusion that in the two cases we have a different state of affairs. In both the nucleus is the ultimate source of the secretory product but in plants it is far more directly concerned than in animals; in the one it is active and in the other passive.

General Conclusions

1. In the Gramineae "diastase of secretion" arises in the nucleus of the epidermal cells of the scutellum. For:

(*a*) At the beginning of germination the nuclei contain dark staining granules, but there are few or none in the cytoplasm.

(*b*) At this same stage, through very small breaks in the membranes of the heavily loaded nuclei, granules are beginning to exude in small streams.

* Green, R., Soluble Ferments and Fermentation, 379.

(*c*) These granules at first spread through the cell but later collect at the end of the cell next to the endosperm, where they are ultimately dissolved.

(*d*) Immediately after their dissolution the first destructive action of a ferment in the cell walls and inter-starch matrix of the endosperm is to be observed.

(*e*) Soon starch grains appear in greater abundance in the scutellar cells.

2. Zymogenesis begins in the nuclei before the advent of the resting period.

3. At some time during the first and third days of germination the diastase-secreting cells swell to three or four times their original size.

4. During the first and third days there are "waves" of secretion, so that this process may be described as intermittent. From that time on, until the final exhaustion of the cells, the secretion is more continuous. (It has been shown by Hansteen * that an accumulation of diastase unless removed will inhibit the further secretion of that substance by the scutellum and it would seem that the behavior of the secreting cells as above described is due to this fact.)

5. The secreting cells begin to degenerate about the tenth day.

6. It is highly probable that the chromatin takes an active part in the zymogenesis.

7. The nucleus is in this case very clearly the metabolic center of the cell.

8. There is a marked difference in secretory processes in plants and animals.

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Explanation of Plate 20

FIG. 1. A somewhat diagrammatic view of a section of a kernel of corn, showing the general relationship of the plantlet, the scutellum, and the endosperm: *end*, endosperm; *ep*, secreting epithelium of scutellum; *pl*, plumule; *r*, radicle; *sc*, scutellum with the vascular tissue. $\times$ 4.

FIG. 2. Diastase-secreting cells before the beginning of germination: *end*, endosperm; *ep*, diastase-secreting cells; *d*, diastase granules; *n*, nucleus; *sc*, scutellar cells; *st*, starch grains. $\times$ 795.

FIG. 3. Shortly after the beginning of germination: *d*, diastase granules. × 795.

FIG. 4. The same cells the latter part of the first day of germination. The firs period of secretion: *d*, diastase granules; *n*, nucleus. × 795.

FIG. 5. The condition of the cells after about three days of germination; corrosion of starch grains; *st*, starch grains; *d*, diastase granules. × 795.

FIG. 6. Diastase-secreting cells during the fifth day of germination. × 795.

FIG. 7. The extremely elongated diastase-secreting cells after about twenty-two days of germination. × 795.

FIG. 8. The corresponding cells of barley during secretion of the enzyme. The seeds had germinated about two days. × 795.

All figures except the first were drawn from camera outlines.